SOYBEAN SELECTION SYSTEM BASED ON AEC-RESISTANCE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001]   This application claims the benefit of International Application No. PCT/US2004/020039, International filing date June 23, 2004, which claims the benefit of U.S. Provisional Patent Application Serial No. 60/483,103, filed on June 30, 2003, the disclosures of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002]   The invention relates to methods of producing a transgenic soybean plant with improved qualities by introducing a dihydrodipicolinate synthase (DHPS) gene conferring S-2-aminoethyl-cysteine (AEC) resistance in soybean somatic embryos as a non-antibiotic selectable marker system. The resulting improved soybean plants eliminate concerns associated with antibiotic resistance genes, and because they are AEC tolerant, they are suitable for agricultural use with AEC as an herbicide.

BACKGROUND OF THE INVENTION

[0003]   Because soybeans are a good source of oil, isoflavones and proteins, genetic engineering of soybeans for higher value cultivars has tremendous market value. Soybean transformation procedures include gene gun bombardment of somatic embryos. The bombarded embryos are selected with a selectable marker that is part of the introduced DNA. For soybean somatic embryo selection, the antibiotic hygromycin is often used. Market forces and health and safety concerns have created a need to eliminate such genes from improved crop varieties, but existing processes are time consuming. There is a need for non-antibiotic selection maker genes that can eliminate these problems. Only a limited number of non-antibiotic resistance markers

are available for plant improvement. For example, herbicide selection systems like glyphosate may be useful but are generally not available for use.

[0004]   Soybean cellular selection systems are exceptional in that they are recalcitrant to selection that commonly works with many plant cellular selection systems. For example, the most common cellular selection system for plant systems is kanamycin resistance conferred by an *nptII* gene, but after numerous failed attempts it was concluded that this selection system cannot be accomplished with soybeans. Antibiotic resistance markers have been used in seeds to produce transgenic canola, tobacco, barley, and soybean plants that express a bacterial variant of DHPS that is insensitive to AEC. The presence of antibiotic resistance genes in these plants is an undesirable result.

[0005]   AEC is a lysine analog that naturally occurs in the mushroom *Rozites caperta* (Matsumoto, 1984; Cadogan et al., 1996). It also can be synthesized using N-(tert-butoxycarbonyl)serine and ethanolamine (Arnold et al., 1988). AEC is an inhibitor of dihydrodipicolinate synthase (DHPS or DHDPS) killing cells and tissues due to an inability to synthesize lysine (Perl et al., 1993; Ghislain et al., 1995; Vauterin et al., 2000). AEC also inhibits AK and lysine is another inhibitor of DHPS (Negrutiu et al., 1984). It is known that the DHPS enzyme of *E. coli* is 50 fold less sensitive to AEC than plant enzymes (IC50 for lysine of 400 µM - 1mM compared to 10 µM for plants.) (Jacobs et al., 2000)

SUMMARY OF THE INVENTION

[0006]   The invention provides a method for making a transgenic soybean plant having no antibiotic resistance gene and improved soybean characteristics by selecting soybean somatic embryos with S-2-aminoethyl-cysteine (AEC   or 2-AEC).

[0007]   This invention relates, *e.g.*, to a method for generating a transgenic soybean plant which comprises in its genome a heterologous nucleic acid sequence of interest, comprising:

[0008] introducing into a soybean somatic embryo a polynucleotide encoding a functional dihydrodipicolinate synthase (DHPS) polypeptide, operably linked to a first expression control sequence, and optionally a polynucleotide encoding a heterologous polypeptide of interest, operably linked to a second expression control sequence, wherein the first and second polynucleotides and expression control sequences may be the same or different, wherein DHPS expressed from the introduced DHPS-encoding polynucleotide is effective to render the embryo resistant to S-2-aminoethylcysteine, and

[0009] contacting the embryo produced in (a) with 2-AEC, under conditions effective to allow an embryo which expresses the DHPS to grow selectably and to mature into a soybean plant, while inhibiting growth of non-transformed embryo cells.

[00010] Progeny of a transgenic soybean plant of the invention include seeds (beans) produced by the plant, as well as variants of the original plant which are produced by further genetic manipulations or crosses. For example, a transgenic soybean plant of the invention may be heterozygous for the DHPS or the heterologous polypeptide of interest; progeny of this plant include plants resulting from genetic manipulations which are homozygous for one of both of those genes.

[00011] The invention also relates to a method for generating a herbicide-resistant transgenic soybean plant comprising: introducing into a soybean somatic embryogenic culture a polynucleotide encoding a functional dihydrodipicolinate synthase (DHPS) polypeptide, operably linked to an expression control sequence, wherein DHPS expressed from the introduced DHPS-encoding polynucleotide is effective to render an embryo resistant to selection-effective amounts of S-2-aminoethylcysteine (2-AEC), and to render the plant resistant to herbicide-effective amounts of AEC, and contacting the embryo with selection effective amounts of 2-AEC.

[00012] The invention also relates to a herbicide-resistant transgenic soybean plant, or progeny thereof, comprising a polynucleotide encoding a functional dihydrodipicolinate synthase (DHPS) polypeptide, operably linked to an expression

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control sequence, wherein DHPS expressed from the introduced DHPS-encoding polynucleotide is effective to render the soybean plant resistant to herbicide-effective amounts of AEC, the plant having no antibiotic resistance marker.

[00013]   The invention relates to a transgenic soybean plant comprising a polynucleotide encoding an AEC resistant DHPS that is expressible in soybean somatic embryos and a polynucleotide encoding a protein imparting a desired trait in the soybean plant or soybean, the plant being free of a polynucleotide encoding a polypeptide imparting antibiotic resistance.

BRIEF DESCRIPTION OF THE DRAWINGS

[00014]   Figure 1 diagrams the biosynthesis pathway of aspartate-derived amino acids.

[00015]   Figures 2a1 to 2a-5 demonstrate that 5 mM AEC can prevent the germination of soybean seeds; 2.5 mM is quite effective. Figures 2b-1 and 2b-2 show that 500 µM AEC completely prevented the germination of soybean somatic embryos; 100 µM AEC was quite effective. Figure 2c shows the use of 0.5 mM, 1.0, mM, 1.5, mM, 2.5 mM and 5.0 mM concentrations of AEC and illustrates that 1.5 mM AEC is sufficient to kill proliferating soybean somatic embryos on 1/5th D20 medium (1/5 MS salts, 1/5 B5 vitamins and 20 mg/L 2,4-D).

[00016]   Figure 2d shows spray studies using major dicot and monocot weeds, pigweed (Amaranthus retroflexus) and giant foxtail (Setaria faberi) in addition to tobacco. These experiments using 7 to 10 day old seedlings show 20 mM AEC is lethal to pigweed, foxtail and tobacco.

[00017]   Figure 3 shows a transformation vector using a Pea RbcS transit peptide to target the *E. coli* DHPS to chloroplasts where the lysine biosynthesis pathway is operative.

[00018]   Figure 4 is a schematic flowchart indicating that soybean somatic embryos were induced from immature zygotic embryos on MS plates containing 40 mg/L 2,4-D. Induced embryos are proliferated either on solid D20 (20 mg/L 2,4-D) or FL Jack liquid medium (10 mg/L 2,4-D). Then green clumps are

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bombarded with DNA coated gold particles using PD-1000 particle bombardment system (a.k.a. "Genegun"). Embryos are selected with 2.5 mM AEC on 1/5 D20 plates. Positive looking green clumps were proliferated, matured. These embryos were stained for GUS and checked for the expression of *E. coli* DHPS gene to confirm the positive transformants. The resulting plants germinate and grow.

[00019]   Figure 5 shows GUS staining of DHPS transgenic embryos.

[00020]   Figure 6 demonstrates the expression of *E. coli* DHPS in different transgenic soybean somatic embryo lines and negative control embryos.

[00021]   Figure 7 shows regeneration of soybean plants via somatic embryogenesis: (a) Immature zygotic embryo cotyledon explants on medium containing 40 mg/L 2,4-D (D40 medium), (b) Somatic embryo induction on D40 medium, (c) Somatic embryo proliferation on D20 medium, (d) Matured cotyledonary stage embryos on medium containing 6% maltose (MSM6), (e) Desiccation of matured embryos, (f) Germination on medium containing 3% sucrose (MSO3), (g) Germinated somatic embryo with well defined root and shoot system, and (h) Regenerated soybean plant transferred to soil.

[00022]   Figures 8a and 8b show protocols for the transformation of soybean by particle bombardment using liquid (8a) and solid (8b) media protocols.

DETAILED DESCRIPTION OF THE INVENTION

[00023]   In describing preferred embodiments of the present invention, specific terminology is employed for the sake of clarity. However, the invention is not intended to be limited to the specific terminology so selected. It is to be understood that each specific element includes all technical equivalents, which operate in a similar manner to accomplish a similar purpose. The embodiments of the invention may be modified or varied, and elements added or omitted, without departing from the invention, as appreciated by those skilled in the art in light of the above teachings. Each reference cited here is incorporated by reference as if each were individually incorporated by reference.

[00024]   As used herein, the term polynucleotide is interchangeable with the terms oligonucleotide, oligomer, and nucleic acid. A polynucleotide of the invention may be a recombinant polynucleotide, a natural polynucleotide, or a synthetic or semi-synthetic polynucleotide, or combinations thereof. Polynucleotides of the invention may be RNA, PNA, LNA, or DNA, or combinations thereof. The nucleotides of a polynucleotide of the invention can be joined via various known linkages, *e.g.*, ester, sulfamate, sulfamide, phosphorothioate, phosphoramidate, methylphosphonate, carbamate, etc., depending on the desired purpose, *e.g.*, improved *in vivo* stability, etc. See, *e.g.*, U.S. Pat. No. 5,378,825. Any desired nucleotide or nucleotide analog can be incorporated, *e.g.*, 6-mercaptoguanine, 8-oxo-guanine, etc.

[00025]   A "functional dihydrodipicolinate synthase (DHPS) polypeptide" exhibits a detectable amount of at least one desirable function or property of a DHPS, such as those having SEQ ID NO: 3 or SEQ ID NO: 4. For example, the expression of the polypeptide renders the organism in which it is expressed (*e.g.*, an *E. coli* bacterium or a soybean somatic embryo or a soybean plant or bean) resistant to growth inhibition by S-2-aminoethylcysteine (2-AEC); and/or it is insensitive to feedback inhibition with lysine. A skilled worker can readily determine whether a given DHPS polypeptide exhibits a detectable amount of a desired function or property, using conventional procedures.

[00026]   The amino acid sequence of a DHPS polypeptide from *E. coli* is AAA23665 [gi:145708] (SEQ ID NO: 3).

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1 mftgsivaiv tpmdekgnvc raslkklidy hvasgtsaiv svgttgesat
lnhdehadvv
61 mmtldladgr ipviagtgan ataeaisltq rfndsgivgc ltvtpyynrp
sqeglyqhfk
121 aiaehtdlpq ilynvpsrtg cdllpetvgr lakvkniigi
keatgnltrv nqikelvsdd
181 fvllsgddas aldfmqlggh gvisvttnva ardmaqmckl
aaeehfaear vinqrlmplh
241 nklfvepnpi pvkwackelg lvatdtlrlp mtpitdsgre
tvraalkhag ll
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[00027]   The amino acid sequence of a wild-type DHPS polypeptide from soybeans is AAA73555.1(gi:548321) (SEQ ID NO: 4).

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1 mitnsaavkp nfhlpmrsfe lknrtspedi kalrlitaik tpylpdgrfd
leayddlvnm
61 qigqgaegvi vggttgegql msweehiili ahtvncfggk ikvigntgsn
streaihate
121 qgfavgmhaa lhinpyygkt sldgmvahfr svlsmgptii
ynvpartgqd ipphviqtla
181 esvnlagvke cvgndrikqy tddgivvwsg nddqchdarw
gygatgvvsv asnlvpglmr
241 elmfggvnpt lnskllplid wlfhmpnpig lntalaqlgv
irpvfrlpfv plpvdkrief
301 anlvkeigre hfvgnkvvev lddddfflvs ry
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[00028]   The amino acid sequence below (SEQ ID NO: 5) is a modified portion of the sequence of SEQ ID NO: 4 in which amino acid 84 is changed to arginine, starting from amino acid 54 of the soybean DHPS. Amino acid 84 is changed to arginine i.e., the 30th amino acid in the sequence shown (SEQ ID NO: 5), indicated by the bold letter "R."

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YDDLVNMQIG   QGAEGVIVGG   TTGEGQLMSR   EEHIILIAHT   VNCFGGKIKV

IGNTGSNSTR   EAIHATEQGF   AVGMHAALHI   NPYYGKTSLD   GMVAHFRSVL

SMGPTIIYNV   PARTGQDIPP   HVIQTLAESV   NLAGVKECVG   NDRIKQYTDD

GIVVWSGNDD   QCHDARWGYG   ATGVVSVASN   LVPGLMRELM   FGGVNPTLNS

KLLPLIDWLF   HMPNP
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[00029]   A "functional" DHPS polypeptide may comprise SEQ ID NO: 3 or SEQ ID NO: 4 modified as shown in SEQ ID NO:5; or it may comprise any of a number of variants of those sequences, either naturally occurring or deliberately generated, provided that the changes do not substantially alter a function or property as discussed above.

[00030]   For example, the functional DHPS polypeptide may be shorter or longer than one of the above polypeptides. In embodiments of the invention, the

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variant DHPS differs from SEQ ID NO: 3 or SEQ ID NO: 4 by one or more modifications, which are either conservative or non-conservative modifications (*e.g.*, insertions, deletions, additions and/or substitutions). By "conservative substitutions" is meant by combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Variants can include, *e.g.*, homologs, muteins and mimetics. Many types of protein modifications, including post-translational modifications, are included. Post-translational modifications include naturally occurring or synthetically produced, covalent or aggregative conjugates with other chemical moieties, *e.g.*, glycosyl groups, lipids, phosphates, acetyl groups, etc., as well as cleavage, such as of terminal amino acid(s). See, *e.g.*, modifications disclosed in U.S. Pat. No. 5,935,835. Other functional variants may comprise added peptide sequences, either naturally occurring or heterologous, such as, *e.g.*, leader, signal, secretory, targeting, enzymatic etc. sequences. In one embodiment, a functional variant DHPS polypeptide exhibits at least about 70% sequence identity (*e.g.*, at least about 80%, 90%, 95%, 98% or 99% sequence identity) to SEQ ID NO: 3 or SEQ ID NO: 4 or SEQ ID NO: 5.

[00031] As *E. coli* DHPS is naturally resistant to 2-AEC no modifications are required. However, with respect to soybean DHPS, as guidance as to which amino acids can be altered without substantially altering the function of the DHPS, the following amino acids of SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5 have been modified to impart lysine resistance to soybean DHPS enzyme (Silk G.W. and B.F. Matthews, 1997, Plant molecular biology, 33:931-933) and can be expected to confer (or to contribute to) resistance to AEC (referring to SEQUENCE ID NO: 4):

[00032] amino acid 104 (ASP)

[00033] amino acid 112 (ALA).

[00034] Changes of other amino acids are less likely to affect this function of the protein, and thus are more amenable to alteration.

[00035] A polynucleotide which encodes a functional variant DHPS of the invention may differ from the sequence of SEQ ID NO: 1 or SEQ ID NO: 2 in a

variety of ways. For example, the sequence may exhibit a percent identity to one of those sequences of at least about 70% (*e.g.*, at least about 80%, 90%, 95%, 98% or 99% sequence identity).

[00036]   The comparison of sequences and determination of percent identity and similarity between two sequences (either polypeptide or polynucleotide) can be accomplished using any of a variety of conventional mathematical algorithms. (Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991).

[00037]   Among the many types of suitable mathematical algorithms are those described in Karlin et al. (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877; the algorithm incorporated into the NBLAST and XBLAST programs (version 2.0) as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402; the GAP program I the GCG software package (Devereux et al. (1984) Nucleic Acids Res. 12 (1):387); and the algorithm of Myers and Miller, CABIOS (1989). Alternatively, a functional variant polynucleotide of the invention may hybridize specifically to a polynucleotide having SEQ ID NO: 1 or SEQ ID NO: 2 under conditions of high stringency. Conditions of "high stringency," as used herein, means, for example, hybridization in a hybridization solution containing, *e.g.*, about 5X SSC, 0.5% SDS, 100 µg/ml denatured salmon sperm DNA and 50% formamide, at 42°C.

[00038]   As used herein, the term "expression control sequence" means a polynucleotide sequence that regulates expression of a polypeptide coded for by a polynucleotide to which it is functionally ("operably") linked. Expression can be regulated at the level of the mRNA or polypeptide. Thus, the term expression control sequence includes mRNA-related elements and protein-related elements. Such elements include promoters, domains within promoters, upstream elements,

enhancers, elements that confer tissue or cell specificity, response elements, ribosome binding sequences, etc. An expression control sequence is operably linked to a nucleotide coding sequence when the expression control sequence is positioned in such a manner to effect or achieve expression of the coding sequence. For example, when a promoter is operably linked 5' to a coding sequence, expression of the coding sequence is driven by the promoter. An expression control sequence may be linked to another expression control sequence. For example, a tissue-specific expression control sequence may be linked to a basal promoter element or to an enhancer that conveys high levels of expression.

[00039]   Any of a variety of expression control sequences can be used in constructs of the invention; suitable expression control sequences will be evident to the skilled worker.

[00040]   In preferred embodiments of the invention, the "first" expression control sequence (regulating the expression of the DHPS sequence) comprises a constitutive promoter, which is expressed constitutively in a wide variety of cell types. Preferably, the promoter is sufficiently strong to produce enough DHPS to generate sufficient levels of lysine to achieve cell growth in the presence of added AEC. Among the suitable strong constitutive promoters/enhancers are expression control sequences from ribosomal RNA promoters or, most preferably, from the CaMV 35S promoter.

[00041]   In embodiments of the invention, the "second" expression control sequence (regulating the expression of the polypeptide of interest) comprises a tissue specific promoter, most preferably a promoter which is active specifically in seeds (a "seed-specific" promoter). Typical examples are glycinin, phaseolin, conglycinin, seed lectin, napin, zein or other seed-specific promoters

[00042]   Both constitutive and tissue-specific promoters isolated from one plant function generally in a wide variety of other plants; thus, expression control sequences from a variety of plant species are suitable for expression in soybeans.

[00043]   In a most preferred embodiment, the "first" expression control sequence (driving expression of the DHPS sequence) comprises a constitutive

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promoter; and the "second" expression control sequence (driving expression of the heterologous polypeptide of interest) comprises a seed specific promoter.

[00044]   In some embodiments of the invention, the DHPS-encoding sequence and/or the sequence encoding the polypeptide of interest are *not* operably linked to an expression control sequence. In such a case, the polypeptide integrates near an endogenous expression control sequence in the plant, and its expression is regulated by that endogenous expression control sequence.   For example, an exogenously introduced polynucleotide may integrate at a random site in the soybean genome, by illegitimate recombination; or a DHPS sequence from *E. coli* may integrate at the site of similar sequences of the endogenous soybean DHPS gene by homologous recombination.

[00045]   Additional coding sequences may be included, such as a transit peptide that targets the polypeptide of interest to go to the desired organelle, such as a chloroplast. For example, Pea RbcS transit peptide is used to target the *E. coli* DHPS to chloroplasts where the lysine biosynthesis pathway is operative.   Other desirable coding sequences will be apparent to a person of ordinary skill in the art.

[00046]   An embryogenic culture comprising the above-discussed sequences encoding DHPS and the polypeptide of interest is "contacted" by the AEC by any of a variety of conventional means.   These include, *e.g.*, incorporation of AEC into the liquid or solid culture media. Methods for allowing the embryos to grow to form plants and to pollinate and produce seeds (beans) are conventional in the art.

[00047]   In preferred embodiments of the invention, the DHPS and heterologous nucleic acids are stably integrated into the genome of the plant. However, in other embodiments, these nucleic acids are introduced into the embryo on minichromosomes and are maintained stably, and are transmitted through the germ line.

[00048]   "Genome" is intended to refer to the chromosomes in the nucleus of the soybean plant but also any autonomously replicating DNA that is passed to daughter cells, e.g. in chloroplasts and mitochondria.

[00049]   Selection effective conditions are those that inhibit growth of non-resistant embryos, and are effective to grow transformants selectably, e.g. a concentration and formulation of AEC sufficient to slow the growth of non-transformed embryo cells while permitting transformed embryo cells to grow better than non-transformed embryo cells, under the same conditions, preferably under conditions where the non-transformed cells do not grow at all and transformed cells grow well, e.g. an AEC concentration of 1 mM.

[00050]   In a preferred embodiment of the invention, the polynucleotide encoding the DHPS polypeptide (which is operably linked to the first expression control sequence) and the polynucleotide encoding the polypeptide of interest (which is operably linked to the second expression control sequence) are on the same plasmid. The sequence encoding the functional DHPS polypeptide may be either upstream or downstream of the sequence encoding the heterologous polypeptide of interest. In another embodiment, the two polynucleotides are on separate plasmids.

[00051]   A wide variety of heterologous "polypeptides of interest" will be evident to the skilled worker. These can be, e.g., "input" genes (such as genes conveying herbicide resistance, etc.) or "output" genes (such as genes that provide a nutritional trait, increased oil content, proteins, etc.) Input traits are traits that benefit growers such as better or easier weed control, insect or disease resistance etc. Output traits benefit processors or consumers such as healthier or more stable oil, protein higher in limiting amino acids such as methionine, lower in oligosaccharides or phytate, etc. Among suitable polypeptides of interest are, e.g., omega-3 desaturase; a polypeptide providing improved meal amino acid compositions; a polypeptide imparting resistance to a bacterium, a fungus, a virus, an insect, or a nematode; a polypeptide providing herbicide resistance; a polypeptide affecting soybean composition or quality; a polypeptide that enhances nutrient utilization; or a polypeptide providing resistance to environmental factors (such as drought) or stress. More particularly, the polypeptide can be phosphinothricin acetyltransferase, glyphosate resistant EPSPS, aminoglycoside phosphotransferase, hygromycin phosphotransferase, neomycin phosphotransferase, dalapon dehalogenase,

bromoxynil resistant nitrilase, anthranilate synthase or glyphosate oxidoreductase. Other preferred heterologous polypeptides of interest include a lysophosphatidate acyl transferase (LPAT); a diacylglycerol acyltransferase (DGAT); a polypeptide which provides increased oil content in the soybean; delta-9 desaturase (which can result in a decreased saturated fatty acid contents in the soybean plant, resulting in palmitoleic acid accumulation in the soybean plant); and delta-12 desaturase (which can result in high oleic acid content soybean oil); and DHPS.

[00052] The DHPS-encoding sequence which is operably linked to the first expression control sequence may take any of a variety of forms. Generally, the sequence encodes a DHPS that is resistant to AEC inhibition, such as a sequence isolated from a bacterium (*e.g.*, *Escherichia coli*), or a sequence isolated from *Corynebacterium glutamicum* or *Nicotiana sylvestris*. In a preferred embodiment, the DHPS-encoding sequence is isolated from *E. coli*, and is represented by SEQ ID NO: 2. In other embodiments of the invention, the DHPS-encoding sequence is a sequence that has been genetically altered from a wild type sequence to become resistant to AEC inhibition. A variety of possible such alterations are discussed above. A preferred such variant is the mutant version of the endogenous soybean sequence which is discussed in the Examples and whose coding sequence is represented by SEQ ID NO: 1.

[00053] In embodiments of the invention, a 3' terminator sequence (*e.g.*, a polyA addition and/or a RNA cleavage sequence) is located 3' to one or both of the DHPS-encoding sequence and the sequence encoding the heterolgous polypeptide of interest. The skilled worker will be aware of a number of suitable terminator sequences. These include, *e.g.*, a pea RUBISCO 3' controlling sequence, a ribosomal RNA terminator, or a 3' transcription region for the nopaline synthase (NOS) gene.

[00054] In embodiments of the invention, one or both of the sequence encoding the functional DHPS polypeptide and the sequence encoding the heterologous polypeptide of interest is operably linked to two or more expression control sequences. For example, a minimal promoter and some sort of enhancer would normally be needed. In embodiments of the invention, a transgenic soybean plant

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produced as above is further backcrossed to generate a transgenic soybean plant which is homozygous for the sequence encoding the heterologous polypeptide of interest. If the gene encoding the polypeptide of interest is dominant, it is, of course, not necessary for the gene to be homozygous in order for it to express functional proteins. However, because soybean plants are generally sold as inbred lines, which breed true, it is preferable that the plants (and seeds derived therefrom) be homozygous for these heterologous genes. Methods for performing suitable backcrosses are conventional, and suitable crosses will be evident to the skilled worker.

[00055] In embodiments of the invention, suitable backcrosses are performed in order to generate a transgenic soybean plant which is homozygous for the sequence encoding the DHPS polypeptide. Homozygosity of these genes is not required for future growth of plants (or seeds therefrom) which express heterologous polypeptides of interest. In fact, the selectable marker at this stage can even be absent. However, as discussed more fully below, in some embodiments of the invention, DHPS is expressed constitutively, at high levels, in order to protect the plant against inhibition by AEC, thereby allowing the use of AEC as a pesticide. In these embodiments, it is preferable that the DHPS is homozygous in the plant.

[00056] In embodiments of the invention, the transgenic plant is fertile. However, in other embodiments (*e.g.*, when the leaves are to be used), the plant does not need to be fertile.

[00057] The method may include using a transformation vector, which comprises the DHPS coding sequence, an appropriate expression control sequence (such as a strong constitutive promoter such as the 35S promoter) and optionally, a terminator/3' controlling region. The vector also comprises a gene of interest for soybean improvement and/or may include other genes for amplification, e.g. a gene for selection in *E. coli,* which can be the DHPS gene itself in an appropriate auxotroph.

[00058] The resultant soybean plant is one expressing a functional DHPS gene that is resistant to AEC levels that inhibit growth of normal soybean cells

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or tissues.   A gene of interest may include lysophosphatidate acyl transferases (LPATs), diacylglycerol acyltransferases (DGATs), both to increase oil contents; delta-9 desaturases to decrease saturated fatty acid contents and/or to achieve palmitoleic acid accumulation; delta-12 desaturases for high oleic acid soybean oil; omega-3 desaturases; genes for improved meal amino acid compositions; genes for disease, insect and nematode resistance; genes for herbicide tolerance; genes for drought resistance and other genes for soybean improvement.

[00059]   The DHPS gene may be a functional bacterial DHPS gene that is resistant or tolerant to AEC.

[00060]   An advantage of the inventive method and plants is that selected plants including AEC-resistant DHPS genes can be resistant to growth inhibitory concentrations of AEC in field use. This permits use of a novel herbicide that can be produced from natural sources, and may be appropriate for organic farming. See Example 4. In particular, this may be used for weed control both in crop fields wherein the crops are selected for resistance to AEC or Lysine and Threonine or around trees, flowers and other plants, where contact to foliage is minimized as well as around buildings and other structures.

[00061]   Examples of suitable DHPS genes include: *Corynebacterium glutamicum (e.g.,* Cremer J, Treptow C, Eggeling L, Sahm H., Regulation of enzymes of lysine biosynthesis in Corynebacterium glutamicum, J Gen Microbiol. 1988 Dec;134 (Pt 12):3221-9, Nucleotide sequence of the dapA gene from Corynebacterium glutamicum; Bonnassie S, Oreglia J, Sicard AM. Nucleic Acids Res. 1990 Nov 11;18(21):6421), *Escherichia coli (e.g.,* Laber B, Gomis-Ruth FX, Romao MJ, Huber R, Escherichia coli dihydrodipicolinate synthase. Identification of the active site and crystallization, Biochem J. 1992 Dec 1;288 (Pt 2):691-5.) or *Nicotiana sylvestris (e.g.,* Ghislain, M., Frankard, V., Jacobs, M. 1995 A dinucleotide mutation in dihydrodipicolinate synthase of *Nicotiana sylvestris* leads to lysine overproduction, *Plant J.* , 8, 733 743).

[00062]   A variety of types of expression control sequences (e.g., promoters) and terminator sequences known to one skilled in the arts that can be

used, such as any strong constitutive or nearly constitutive expression control sequence and terminator. For example expression control sequences may include a 35S promoter or ribosomal RNA gene promoters. Terminator sequences may include pea RUBISCO 3' controlling sequences or Nos terminator sequences.

[00063] A variety of AEC concentrations can be used for the selection of bacterial DHPS transformed soybean somatic embryos, so long as those concentrations are in an amount that would inhibit the growth of soybean seeds or somatic embryos without the AEC resistant DHPS gene. For example, at least about 0.1 mM or higher may be sufficient to inhibit growth, and 1 mM or higher can stop all cell growth. Higher concentrations than about 10 mM may inhibit growth even in transformed embryos. AEC concentrations for selection can be from about 0.5 mM to about 5 mM, 1.0 mM to about 5 mM, 2.5 mM to about 5 mM, or more than or less than about 0.5 mM, 1 mM, 1.5, mM, 2 mM, 2.5 mM, 3.0 mM, 3.5 mM, 4.0 mM, 4.0 mM or 5.0 mM. Concentrations in the range up to about 10 mM or 20 mM may be suitable for selection in some circumstances. In later growth stages and including field use, the higher concentrations of AEC may be preferred, in conjunction with surfactants or other uptake enhancers.

[00064] A variety of transformation protocols may be used with the inventive process. For example, Figures 8a and 8b show the transformation of soybean by particle bombardment using liquid and solid media protocols using somatic embryos (Trick et al., 1997). Once embryogenic cultures are established, one can get plenty of material with a liquid medium based protocol for transformation. However, with older cultures the regenerated plants are mostly sterile (Hazel et al., 1998). With solid medium-based protocols somatic embryos need to induced regularly. The maturation of somatic embryos on MSM6 medium takes much longer than with liquid medium based protocols with FL-Superlite (Samoylov et al., 1998).

[00065] Targeting cells that can give rise to whole plants for transformation and selection of whole plants is quite difficult with soybeans. Kanamycin selection which works well with most plants does not work at all with soybeans. The precise reasons of why some plants such as soybeans are so

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recalcitrant to standard transformation and regeneration systems are unknown, except that it is thought that many more cells in plants such as soybeans are terminally differentiated compared with easier plants such as potatoes or tobacco.

[00066]   Suitable transformation vectors known to one skilled in the art can be used with the inventive process. For example, the Binary vector pCAMBIA-1201, Genbank # AF234293, which may be obtained from Cambia group in Australia. Before cloning pCambia1201, the DHPS gene can be cloned along with pea rbcs chloroplast transit peptide into another binary vector pKalyx. This is done to clone the *E.coli* DHPS gene along with transit peptide under 35S promoter. This vector has a Nos terminator.

[00067]   The accession number is gi|145707|gb|M12844.1 for the following *E. coli* DHPS sequence (SEQ ID NO: 2):

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1 ccaggcgact gtcttcaata ttacagccgc aactactgac atgacgggtg
atggtgttca
61 caattccacg gcgatcggca cccaacgcag tgatcaccag ataatgtgtt
gcgatgacag
121 tgtcaaactg gttattcctt taaggggtga gttgttctta
aggaaagcat aaaaaaaaca
181 tgcatacaac aatcagaacg gttctgtctg cttgctttta
atgccatacc aaacgtacca
241 ttgagacact tgtttgcaca gaggatggcc catgttcacg
ggaagtattg tcgcgattgt
301 tactccgatg gatgaaaaag gtaatgtctg tcgggctagc
ttgaaaaaac tgattgatta
361 tcatgtcgcc agcggtactt cggcgatcgt ttctgttggc
accactggcg agtccgctac
421 cttaaatcat gacgaacatg ctgatgtggt gatgatgacg
ctggatctgg ctgatgggcg
481 cattccggta attgccggga ccggcgctaa cgctactgcg
gaagccatta gcctgacgca
541 gcgcttcaat gacagtggta tcgtcggctg cctgacggta
accccttact acaatcgtcc
601 gtcgcaagaa ggtttgtatc agcatttcaa agccatcgct
gagcatactg acctgccgca
661 aattctgtat aatgtgccgt cccgtactgg ctgcgatctg
ctcccggaaa cggtgggccg
721 tctggcgaaa gtaaaaaata ttatcggaat caaagaggca
acagggaact taacgcgtgt
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781 aaaccagatc aaagagctgg tttcagatga ttttgttctg
ctgagcggcg atgatgcgag
841 cgcgctggac ttcatgcaat tgggcggtca tggggttatt
tccgttacga ctaacgtcgc
901 agcgcgtgat atggcccaga tgtgcaaact ggcagcagaa
gaacattttg ccgaggcacg
961 cgttattaat cagcgtctga tgccattaca caacaaacta
tttgtcgaac ccaatccaat
1021 cccggtgaaa tgggcatgta aggaactggg tcttgtggcg
accgatacgc tgcgcctgcc
1081 aatgacacca atcaccgaca gtggtcgtga gacggtcaga
gcggcgctta agcatgccgg
1141 tttgctgtaa agtttaggga gatttgatgg cttactctgt
tcaaaagtcg cgcctgg
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[00068]   The coding sequence which starts at 272 and ends at 1150 was used.

[00069]   The effect of AEC on proliferating somatic embryos were conducted in both liquid and solid media and methylated seed oil may be added as a surfactant to facilitate AEC absorption. MSO concentration may be about 0.0007%. However, even without MSO, AEC may be effective in growth inhibition of soybean somatic embryos.

[00070]   There are a variety of different sources/suppliers from which to obtain AEC, e.g., Sigma, and there are several published procedures for its synthesis. AEC is a lysine analog that naturally occurs in the mushroom *Rozites caperta* (Matsumoto, 1984; Cadogan et al., 1996) (also, see Example 4).

[00071]   Suitable transformation methods known to one skilled in the art can be used. One of the well established soybean transformation procedures is gene gun bombardment of somatic embryos (Finer and McMullen, 1991). The bombarded embryos are be selected with a selectable marker on the introduced DNA. So far for soybean selection the antibiotic hygromycin is the one mostly in use. Known methods are adapted to eliminate antibiotic resistance genes commonly included on existing vectors, while permitting amplification (growth) of the vector plasmids. For example, gene gun transformation has traditionally used the entire cloning plasmid

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including an antibiotic resistance gene for selection in *E. coli* but a restriction site can be included so that after amplification, the antibiotic resistance gene section can be cut out, leaving the DHPS gene and the gene of interest to be used for bombardment into the cells.

[00072]   For another example, with Agrobacterium mediated transformation normally only DNA between the T-left and T-right borders is incorporated into plant chromosomes. Accordingly, the antibiotic resistance gene(s) for bacterial selection can be placed outside these borders so that they are not inserted into the target cells by the transformation process.

[00073]   For a third example, one can flank genes that are not wanted in post-transformation target plant chromosomes with CRE sites, so that crossing the resulting transgenic plants containing inserted LOX genes will cause the genes flanked with CRE sites to be cleaved out in subsequent generations.

[00074]   No antibiotic resistance genes are needed for cloning in *E. coli* auxotrophic for DHPS. A preferred method is to amplify such antibiotic-resistance gene-free vectors in *E. coli*, and then use gene gun bombardment for transformation.

[00075]   An improved soybean seed according to the invention can be planted in an open field. AEC is applied during germination, growth, and/or maturation to eliminate weeds. Soybeans are harvested from the mature plant, having improved output traits. The DHPS gene used to confer AEC resistance can be derived from a soybean, and the AEC that is used can be obtained from natural sources, permitting the harvested soybeans to be marketed more readily than transgenic soybeans having heterologous genes, antibiotic resistance genes, or glyphosate or other herbicide resistance genes that require use of synthetic herbicides.

EXAMPLES

EXAMPLE 1

[00076]   As a first step in the selection, different concentrations of AEC were used to see if it can kill the proliferating soybean somatic embryos as well as tobacco leaves and germinating tobacco and soybean seeds and germinating soybean

somatic embryos. The effect of AEC is clearly seen in all these cases with varying concentrations. 100 μm AEC is sufficient to completely inhibit the germination of soybean somatic embryos whereas for germinating soybean seeds and tobacco seeds 2.5 mM AEC is required. For proliferating soybean somatic embryo concentrations up to 1 mM AEC is needed along with 0.0075% methylated seed oil to kill them. To further confirm that AEC is acting on the above mentioned tissues, the *E. coli* DHDPS gene was cloned and put into the binary vector p1201 with the pea RBCs transit peptide under the CaMV 35S promoter.

[00077] Proliferating soybean somatic embryos were shot with a gene gun using the *E. coli* DHDPS gene construct and the embryos were selected on both solid D20 and liquid Fl cv Jack proliferating media with 1 mM to 2.5 mM AEC with 0.0075% methylated seed oil. The positive looking green clumps were put on the MSM6AC maturation medium. Some of the embryos have been shown to contain an introduced GUS gene indicating the soybean transformation with AEC as a selective agent can be achieved.

EXAMPLE 2

[00078] **Lysine-Threonine and Lysine-analog S-(2-aminoethyl)-L-cysteine as Alternative Non-antibiotic Selection System for Soybean somatic Embryos.**

Introduction

[00079] Plant cells and tissues are normally killed by exposure to S-2-aminoethyl-cysteine (AEC), which prevents lysine synthesis through inhibition of the plant enzyme dihydrodipicolinate synthase (DHPS). *E. Coli* DHPS is not inhibited by AEC. A vector for producing transgenic soybean plants encodes *E. Coli* DHPS. Soybean cells and tissues that are successfully transfected with the vector can be identified by exposure to AEC, which kills cells and tissues not containing the gene encoding *E. Coli* DHPS.

Procedure

[00080]   An AEC selection system for soybean seeds with precise genetic changes and model system seeds (for rapid system validation) were germinated on 2 mM and 5 mM AEC containing agar plates. On the control plates without AEC all the seeds germinated, however only ~ 10% of the soybean seeds germinated on 2 mM AEC plates and none on 5 mM plates. Not a single model system seed germinated at either of these concentrations.

[00081]   The effect of AEC on soybean somatic embryos were checked at two stages (1) during the germination (2) during proliferation. The AEC effect during germination was very effective with concentrations above 100 μM were enough to prevent the germination completely.

[00082]   However, selection during proliferation is always a better way as selected clones can be proliferated and the untransformed embryos can be killed in the earlier stages itself. Unfortunately concentrations up to 20 mM were not sufficient to kill most of the proliferating embryos, though some of the embryos turned yellowish and developed brown spots, but many of them continued to proliferate like controls even after two changes of the medium, once every 15 days. To find out if surfactants can help in AEC absorption during proliferation a methylated seed oil based surfactant was used. A series of experiments were run with liquid proliferation medium to determine a suitable surfactant concentrations as the 0.2% which was used initially killed the cells even without AEC addition to the medium. It was determined that a 0.005% concentration is suitable to use with AEC. However on solid medium with 0.2% surfactant both controls and 10 mM AEC cultures remained green with growth for two weeks before they died. The cultures on plates with 20 mM AEC died in 4 days indicating solid medium selection can be useful.

[00083]   To test if the modified soy DHDPS can become insensitive to AEC and whether 18:1 desaturases can function as a 16:1 desaturase, experiments were conduced with *E. coli* auxotrophs. A DHDPS auxotroph was obtained from the

E. coli genetic center that can be rescued on medium without diaminopimelic acid when transformed with lysine insensitive DHPS and not with the wild type gene. Similarly, the *E. coli* auxotroph (fabA/fadR mutant) was obtained which can be complimented by C_{14} and C_{16} ACP desaturases but not by stearoyl-ACP desaturases. The primers required for cloning were designated and obtained from soybean genes. A suitable *E. coli* expression vector was obtained to express these genes in the respective auxotrophs.

Summary

[00084]   Experiments were conducted with different tissues of soybean to determine whether LT and AEC can be used as selection agents. Both AEC and LT can inhibit seed as well as soybean somatic embryos (SSE) germination. AEC concentrations of 1.5 to 2.5 mM are effective for selection in presence of 0.075% MSO (methylated seed oil) as surfactant. However LT (up to 10 mM) were not able to inhibit growth of proliferating SSE. Soybean SE and tobacco transformed with wild-type *E. coli* DHPS are found to be resistant to normally lethal concentrations of AEC. Green proliferating transgenic somatic embryos were found to be selected at the concentration level of 2.5 mM AEC. Similarly, selection at low micro molar AEC and 1.5 mM LT resulted in transgenic tobacco shoots. These transgenic SS embryos and tobacco shoots were also observed to be GUS positive indicating resistance to AEC can be used as a selection system for soybean and tobacco.

Results

[00085]   To determine whether AEC can be developed as a selectable marker for soybeans the effect of different concentrations of AEC was examined on the germination of soybean seeds and matured soybean somatic embryos and on proliferating soybean somatic embryos. The effect of AEC is clearly seen in all these cases with varying concentrations. The effect of AEC was also examined as a herbicide against pigweed, foxtail and tobacco.

[00086]   Figures 2a1 to 2a-5 demonstrate that 5 mM AEC can prevent the germination of soybean seeds; 2.5 mM is quite effective. Figures 2b-1 and 2b-2

show that 500 μM AEC completely prevented the germination of soybean somatic embryos; 100 μM AEC was quite effective. Figure 2c shows the use of 0.5 mM, 1.0, mM, 1.5, mM, 2.5 mM and 5.0 mM concentrations of AEC and illustrates that 1.5 mM AEC is sufficient to kill proliferating soybean somatic embryos on 1/5th D20 medium (1/5 MS salts, 1/5 B5 vitamins and 20 mg/L 2,4-D). Figure 2d shows spray studies using major dicot and monocot weeds, pigweed (Amaranthus retroflexus) and giant foxtail (*Setaria faberi*) in addition to tobacco. These experiments using 7 to 10 day old seedlings show 20 mM AEC is lethal to pigweed, foxtail and tobacco.

[00087]   Figure 3 shows a transformation vector using Pea RbcS transit peptide to target the *E. coli* DHPS to chloroplasts where the lysine biosynthesis pathway is operative.

[00088]   In Figure 4, SS embryos were induced from immature zygotic embryos on MS plates containing 40 mg/L 2,4-D. Induced embryos are proliferated either on solid D20 (20 mg/L 2,4-D) or FL Jack liquid medium (10 mg/L 2,4-D). Then green clumps are bombarded with DNA coated gold particles using PD-1000 particle bombardment system (a.k.a. "Genegun"). Embryos are selected with 2.5 mM AEC on 1/5 D20 plates. Positive looking green clumps were proliferated, matured. These embryos were stained for GUS and checked for the expression of *E. coli* DHPS gene to confirm the positive transformants.

[00089]   Figure 5 shows GUS staining of DHPS transgenic embryos.

[00090]   Figure 6 demonstrates the expression of *E. coli* DHPS in different transgenic soybean somatic embryo lines and negative control embryos.

Discussion

[00091]   The AEC selection results showed that AEC can be successfully used as a selection marker for soybeans. The added advantage of this selection system besides being a non-antibiotic selection marker is that it could increase the levels of essential amino acid lysine. No problems were faced in the maturation and germination of these transgenic embryos. When the effect of AEC was tested at the whole plant level, 20 mM AEC was sufficient to kill fox tail and tobacco and effectively prevented the growth of pig weed.

Conclusion

[00092]   The lysine analog AEC is a successful non-antibiotic selectable marker for the transformation of soybean somatic embryos.

EXAMPLE 3

SEQ ID No. 1:

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ctttggactctgaagctatgatgacttggtgaatatgcagattggacaag
gggctgaaggtgttattgttggtgggacaactggtgaaggccaattaatg
agccgggaagagcacataatacttattgctcatacagtcaactgttttgg
tgggaaaattaaggttattggaaatactggaagcaactccaccagggaag
caattcatgccactgagcagggttttgctgttggaatgcatgctgccctt
cacataaaccttactatggcaaaacctccttggatggtatggttgctca
ctttcgaagtgtgctttccatgggacccacaataatctacaatgtgcctg
cacggaccggacaagacattcctccgcatgtaattcaaaccttagctgaa
agtgttaacctggctggtgtcaaggagtgtgtgggaaatgaccgaatcaa
acagtatacagatgatggaattgttgtgtggagtgggaatgatgatcaat
gtcatgatgctagatggggttatggggctaccggagtggtatctgttgcg
agcaacctggttcccggtttaatgcgagaactcatgtttggcggtgtaaa
ccctactctaaattctaaactcttgcctctgattgactggcttttccaca
tgccaaaccccatnggtttgaacactgctcttgctcaacttggggncatc
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[00093]   The above sequence was derived from the wild-type soybean sequence encoding the polypeptide of SEQ ID NO:4.   The nucleotide sequence shown as SEQ ID NO:1 corresponds to sequences encoding the polypeptide of SEQ ID NO:5, a genetically altered AEC resistant DHPS sequence derived from soybean. The soybean DHPS sequence shown as SEQ ID NO:1 was modified from the wild-type to comprise a change of tgg to cgg at the indicated position in bold type. According to the invention, a soybean DHPS coding sequence comprising the indicated modification provides the desired AEC resistance.

[00094]   The sequence was prepared using techniques well know to one skilled in the art. (e.g., Vauterin M, Frankard V, Jacobs M., Functional rescue of a bacterial dapA auxotroph with a plant cDNA library selects for mutant clones encoding a feedback-insensitive dihydrodipicolinate synthase, Plant J. 2000 Feb;21(3):239-48.; and Shaver, J., Bittel, D., Sellner, J., Frisch, D., Somers, D., Gengenbach, B. 1996 Single-amino acid substitutions eliminate lysine inhibition of maize dihydrodipicolinate synthase. *Proc. Natl Acad. Sci. USA* , **93**, 1962 66).

[00095]   It was shown in Arabidopsis, and populous deltoidsXpopulus tricho carpa modification of tryptophan53 (W) to Arginine53 (R) made the DHPS enzyme from these two plants insensitive to lysine concentrations up to 1mM. *E. coli* auxotroph AT997 was rescued using these modified genes. This tryptophan residue is present in the consensus sequence MSWDEHI (SEQ ID NO: 6) in several plant DHPS genes (Vauterin et al.,2000). Clones expressing the insensitive plant DHPS enzymes were resistant to 2.5mM 2-AEC. With our modified soybean DHPS gene we obtained similar results. We also targeted the tryptophan residue present in the consensus sequence MSWDEHI (SEQ ID NO: 6) in the soybean DHPS gene to convert it to arginine. With the modified gene we rescued the E.coli auxotroph AT998 and found that the rescued auxotroph grows well on minimal media plates with 0.5mM 2-AEC concentrations. Native soybean DHPS gene failed to rescue the auxotroph while the modified soybean DHPS gene and native E.coli DHPS were successful in rescuing the mutant (data not shown).

EXAMPLE 4

Method for Weed Control

[00096]   The purpose of this example is to illustrate a method for weed control that has several potential advantages over existing methods.

Background:

[00097]   The broadest class of major herbicides is the acetolactate synthase (ALS) inhibitors including the sulfonylureas, imidazolinones, sulfonamides and pyrimidinylthiobenzoate. DuPont and Syngenta have commercialized no less than 12 different sulfonylureas that target ALS. American Cyanamid (now BASF) have at least 6 different imidazolinones herbicides on the market all of which also target ALS (Shaner and O'Connor, 1991). ALS catalyzes the first step in the biosynthesis of the essential amino acids valine, isoleucine and leucine, as set forth in Figure 9.

[00098]   Via a mutagenesis program, DuPont has been able to produce an altered ALS in soybeans that is resistant to sulfonylureas and from this have developed sulfonylurea tolerant soybeans (STS) (Sebastian, 1990).

[00099]   Roundup (glyphosate) also functions as an amino acid biosynthesis inhibitor targeting 5-enoylpyruvylshikimate 3-phosphate (EPSP) blocking aromatic amino acid biosynthesis, as shown in Figure 10.

[000100]   All of these herbicides kill plants by starving them of essential amino acids but do not affect animals as essential amino acids in animals are obtained via their diets.

[000101]   The selective agent AEC, the chemical structure of which is shown in Figure 11, can be synthesized using N-(tert-butoxycarbonyl)serine and ethanolamine (Arnold et al., 1988).   AEC is an inhibitor of dihydrodipicolinate synthase (DHPS) killing cells and tissues due to an inability to synthesize lysine (Perl et al., 1993; Ghislain et al., 1995; Vauterin et al., 2000). AEC also inhibits AK and lysine, which is also an inhibitor of DHPS (Negrutiu et al., 1984). AEC is shown below to be a more effective more effect herbicidal agent than lysine or lysine + threonine.

Results:

[000102]   A major dicot and monocot weed of tobacco, pigweed (*Amaranthus retroflexus*) and giant foxtail (*Setaria faberi*) was used in addition to a model crop plant, tobacco. Initial experiments using 7 to 10 day old seedlings showed 10 mM lysine and threonine and above is lethal to pigweed, foxtail and tobacco. For larger plants grown in the green house 20 mM lysine and threonine or 40 mM lysine alone are needed for effective weed control. To increase uptake of these compounds, up to 0.2% Silwett (surfactant) was used. Silwett at 0.5% and above kills the plants. Silwett was not found to be a very effective surfactant. Studies with cultured cells and seedlings indicate that 100 μM levels of AEC are effective for selection.

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